

Standardization of Loop mediated isothermal amplification for detection of *D. nodosus* and *F. necrophorum* causing footrot in sheep and goats

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Research Article

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Abstract

The Loop Mediated Isothermal Amplification (LAMP) was standardized for rapid detection of *D. nodosus* and *F. necrophorum*. A total of 250 foot swabs (200) were screened from sheep and (50) were from goats from different districts of Rayalaseema viz., Chittoor, Nellore, Kadapa, Anantapur. Out of 250 samples 75 (30.0%) and 85 (34.0%) were positive for *D. nodosus* and *F. necrophorum* respectively. All the 250 samples were screened individually for both the organisms by LAMP. Among them, 104 (41.6%) were found to be positive for *D. nodosus* and 120 (48.0%) were positive for *F. necrophorum*. The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR by using standard dilutions of DNA extracted from *D. nodosus* and *F. necrophorum* cultures. The detection limit was found to be higher than PCR for both the organisms. The sensitivity of LAMP is compared with PCR by targeting 16S rRNA gene of *D. nodosus* and *lktA* gene of *F. necrophorum*. In case of *D. nodosus*, out of 250 samples, 75 (30.0%) were positive by PCR and 104 (41.6%) were positive by LAMP. Among 250 samples, 85 (34.0%) were positive by PCR and 120 (48.0%) were positive by LAMP in case of *F. necrophorum*. The LAMP was found to be more sensitive than PCR in detecting the organisms with high statistical significance.

Introduction

livestock is the major contributing factor for India being a developing country which mainly depends on agriculture as the main source of income. Sheep and goats play an important role in the livelihood of many small and marginal farmers and landless laborers. Footrot has been reported previously from temperate climates of Jammu and Kashmir alone for the last 18 years. In India, The disease was reported later on from unusual tropical climate of Andhra Pradesh and Tamil Nadu states of southern India. Outbreaks of footrot were being reported regularly from Andhra Pradesh affecting sheep and goat population (Wani *et al.* 2007; Farooq *et al.* 2010; Thomas *et al.* 2011; Sreenivasulu *et al.* 2013; Kumar *et al.* 2013; Kumar *et al.* 2015; Kumar *et al.* 2016). At present, footrot is endemic disease in the states of Jammu and Kashmir, Uttar Pradesh and Himachal Pradesh, Andhra Pradesh.

Footrot is mainly caused by the synergistic action of *Dichelobacter nodosus* formerly (*Bacteroides nodosus*) as the primary transmitting agent, *Fusobacterium necrophorum* as secondary pathogen. Both are anaerobic, gram-negative, rod shaped bacterium with characteristic knobs at each end and is fimbriated (Sreenivasulu *et al.* 2013). Clinically severe disease is referred as the virulent footrot, while milder mainly interdigital form of the disease refers to benign footrot, based on the production of thermostable proteases or the integrase A gene (*intA*), which relate to the corresponding forms of the disease (Cheetham *et al.* 2006). The term intermediate footrot is used to refer forms of footrot that are in between the two forms. The organism *Dichelobacter nodosus* was classified as 10 serogroups (A-I and M) identified based on K-type agglutination of the surface antigens. These serogroups are further divided into 19 serotypes based on cross absorption tests. Type IV fimbriae and extracellular proteases are essential for virulence of *D. nodosus* (Kennan *et al.* 2001; Kennan *et al.* 2010). Class I fimbriae included those of serogroups A, B, C, E, F, G and I, while class II contained in serogroups D and H. The two sets of fimbriae are also distinguished by their disulphide loop profile Elleman (1988).

Fusobacterium necrophorum subsp. *necrophorum* produce a secreted protein leukotoxin (*lktA*) a major virulence factor along with other potent virulence factors such as leukotoxin, lipopolysaccharide and hemagglutinin (Tan *et al.* 1992; Amoako *et al.* 1993; Narayanan *et al.* 2001; Amit Kumar *et al.* 2013).

Diagnosis of footrot disease by isolation of the organism, followed by antigenic analysis using serological procedures may take 3-4 weeks. Alternatively PCR based methods without need to culture have been used for initial detection (La Fontaine *et al.* 1993) and characterization (Dhungyel *et al.* 2002) of *D. nodosus*. Nucleic acid amplification by PCR is one of the most valuable alternative for the detection of *D. nodosus* causing footrot. High precision instruments and standardized protocols are essential for PCR based methods for detection and confirmation of the amplified products. In addition, PCR is not suitable to adopt for routine clinical use. The loop-mediated isothermal amplification (LAMP) assay may fulfill all the above parameters for which it can be used as low cost alternative for detection of organisms causing footrot.

Materials And Methods

Collection of samples

A total of 250 footswabs were collected from the flocks of sheep and goats in different districts of Rayalaseema regions (Chittor, Kadapa, Nellore, Ananthapur) where the prevalence of footrot is high (Table 1). Among them 200 were collected from sheep and 50 are from goats. Samples were collected from the animals showing the symptoms like lameness, decreased body weight, infectious dermatitis of the inter digital skin with a grey scum, pungent and characteristic rotting smell.

Table 1 Details of Samples collected

S.No	Name of the district	No. of flocks visited	No. of samples collected		
			Sheep	Goats	Total
1	Chittor	40	107	15	122
2	Kadapa	15	38	9	47
3	Nellore	6	35	12	47
4	Ananthapur	5	20	14	34
	Total	66	200	50	250

DNA extraction by boiling method

Swabs collected from the interdigital space of the hooves were suspended in 150µL of sterile phosphate buffered saline (PBS) and the suspension was prepared by gentle vortexing for five minutes. The suspension was boiled for ten minutes and then immediately cooled down on ice for 10 minutes. The

suspension was centrifuged at 10,000rpm for ten minutes. The supernatant was used as DNA. The supernatant along with debris was stored at -20°C for further use. Two microlitre of sample was used as template in PCR (Kumar *et al.* 2015).

Reference strain

The *F. necrophorum* DNA was provided by UNIVERSITY OF WARWICK, London, United Kingdom with Ref no: Fn DSM 21784. *D. nodosus* DNA was prepared from stock cultures (serogroup I) maintained in this laboratory.

Screening of clinical samples by Duplex PCR

DNA was extracted by boiling method and all the samples were screened by duplex PCR for the presence of the 16S rRNA of *D. nodosus* and *IktA* gene of *F. necrophorum*. The duplex PCR was performed using a thermocycler with a final reaction volume of 25 µL containing 2.5 µL of Taq buffer (10x); 1 µL of 25 Mm Mgcl₂; 0.3 µL of d NTP mix (10mM); 0.6µL of 16S rRNA (F+R) (10pmol); 0.6µL of *IktA* (F+R) (10pmol); 0.5 µL of *Taq* DNA polymerase (5U/µL); 3.0µL of Template DNA and 16.5 µL of Nuclease free water. Amplification was obtained with 35 cycles, following the PCR 10 µL of amplified products was confirmed by using gel electrophoresis in a 1.5% agarose gel. The amplified bands were visualized under UV illumination (Harsha, 2019).

Primer designing for LAMP

The LAMP primers targeting *D. nodosus* 16S rRNA and *IktA* gene of *F. necrophorum* were designed by using the LAMP primer design software program Primer Explorer V5, from Ekin Chemical Company, Japan(<http://primerexplorer.jp/elamp3.0.0/index.html>). A set of four AT rich primers comprising two outer and two inner primers were designed. The two outer primers were known as the forward outer primer (F3) and the backward outer primer (B3) which helps in strand displacement. The inner primers were known as the forward inner primer (FIP) and the backward inner primer (BIP), respectively. FIP contains F1C (complementary to F1), a TTTT spacer and the F2 sequence. BIP contains the B1C sequence (complementary to B1), a TTTT spacer and B2 sequence. The primer details were given in the (Table 2 and Table 3). The primers were procured from Eurofins Genomics India Pvt. Ltd., Bangalore.

Table 2 Primers for *D. nodosus* used in LAMP reaction

Label	5'position	3'position	Length	Tm	GC%	Sequence
F3	998	1017	20	60	0.55	CCTTCGGGA ACTCTGAGACA
B3	1163	1181	19	59	0.53	GGCCATGATGACTTGACGT
FIP			39			CGCTCGTTGCGGGACTTAACC GGTGTTCATGGCTGTCG
BIP			41			CTTGCCAGCACGTAAAGGTGGG CACCTTCCTCCGGTTTGTC
F2	1018	1035	18	60	0.61	GGTGTTCATGGCTGTCG
F1c	1059	1079	21	65	0.62	CGCTCGTTGCGGGACTTAACC
B2	1138	1156	19	59	0.58	CACCTTCCTCCGGTTTGTC
B1c	1095	1116	22	65	0.59	CTTGCCAGCACGTAAAGGTGGC

Table 3 Primers for *F. necrophorum* used in LAMP reaction

Label	5'position	3'position	Length	Tm	GC%	Sequence
F3	1523	1543	21	5.62	0.38	AGACAGTGTCAATGAGGAAAT
B3	1717	1734	18	56.01	0.50	TTCGTACAGCCACACTTC
FIP			48			TGCCACAGAAGCAGAATTATTATGACTA ACAGACTTATTTAGTGTCGG
BIP			42			GAAGACTTTCTTCAGGAGTGGAAGCACT TGACGTAGTCGCTC
F2	1554	1576	23	56.04	0.39	CTAACAGACTTATTTAGTGTCGG
F1c	1598	1622	25	60.68	0.36	TGCCACAGAAGCAGAATTATTATGA
B2	1699	1716	18	56.43	0.56	CACTTGACGTAGTCGCTC
B1c	1636	1659	24	60.65	0.46	GAAGACTTTCTTCAGGAGTGGAAG

Standardization of LAMP reaction

LAMP reaction was standardized for *16S rRNA* of *D. nodosus* and *lktA* gene of *F. necrophorum*. The enzyme *Bsm* DNA polymerase was procured from Thermo Scientific, Inc. The LAMP was standardized to determine optimum concentrations of primers, enzyme, temperature and time combinations for amplification of target

genes. For *D. nodosus* a total volume made up to 25 µl using nuclease free water with MgCl₂ (25 mM)-1 µl; *Bsm* buffer (×10)-2.5 µl; dNTP mix (10 mM)-7.5 µl; *Bsm* DNA polymerase (8 U/µl) – 0.3 µl; temple (target DNA) – 2 µl; F3 (10 pmol/µl) – 1 µl; B3 (10 pmol/µl) – 1 µl; FIPα (40 pmol/µl) - 4 µl; BIP (40 pmol/µl) - 4 µl; nuclease-free water – 2.2 µl. In case of *F. necrophorum* a reaction mixture of 25 µl was prepared by using MgCl₂ (25 mM)-1 µl; *Bsm* buffer (×10)-2.5 µl; dNTP mix (10 mM)-7.5 µl; *Bsm* DNA polymerase (8 U/µl) – 0.5 µl; temple (target DNA) – 2 µl; F3 (10 pmol/µl) – 1 µl; B3 (10 pmol/µl) – 1 µl; FIPα (40 pmol/µl) - 4 µl; BIP (40 pmol/µl) - 4 µl; nuclease-free water – 2 µl.

LAMP amplification

The LAMP amplification was done in thermal cycler by the following steps with negative controls added with nuclease free water in place of template DNA. In the initial reaction nuclease free water, *Bsm* buffer (×10), dNTP smix and template were added into ependorff tube with initial denaturation at 95°C for 5 min. After chilling the samples for 30 s to 1 min, outer primers, inner primers and enzyme were added. For *D. nodosus* LAMP reaction was standardised at 60°C for 60 min was shown in Fig. 1 followed by enzyme inactivation step for 10 min at 80°C. In case of *F. necrophorum* the LAMP reaction was standardized at 56°C for 60 min was shown in Fig. 2. The amplified LAMP products were stored at -20°C.

Electrophoresis of LAMP products

LAMP products were subjected to agarose gel electrophoresis in a Genei submerged gel apparatus. Agarose gels were prepared with 2.0% agarose using 1xTBE buffer (Tris buffer, Boric acid and EDTA). The LAMP products were mixed with 2µL of 6x gel loading dye and 10µL of the products were mixed and loaded in each well. Electrophoresis was carried 80volts for 45min at 35mA. Electrophoresis was stopped when the dye front reached two-third of the gel. Gel was visualized under ultraviolet (UV) transilluminator and photographed with gel documentation system (Alpha Innotech, Alphaimager, HP).

Comparison of efficacy of LAMP with PCR

The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR using the standard doubling dilutions of *D. nodosus* culture DNA and *F. necrophorum* DNA provided by UNIVERSITY OF WARWICK, London, United Kingdom with Ref no: Fn DSM 21784. The dilutions were performed with initial ten-fold dilution followed by two-fold dilutions.

Comparison of sensitivity of PCR and LAMP

All the 250 field samples were tested with LAMP and the results were compared with duplex PCR results. The diagnostic sensitivity of the LAMP was compared with duplex PCR results using Chi square analysis. There is a significant level of difference between the two tests by this it can be concluded that LAMP is more sensitive than PCR in detecting and screening of the clinical samples.

Table 4 Comparison of diagnostic sensitivity of LAMP with PCR

	PCR	LAMP	Total
<i>D. nodosus</i> positives	75	104	179
<i>D. nodosus</i> negatives	175	146	321
Total	250	250	500
The chi- square statistic with Yates correction is 7.318. The p- value is 0.006827. Significant at p < .05 level.			
<i>F. necrophorum</i> positives	85	120	205
<i>F. necrophorum</i> negatives	165	130	295
Total	250	250	500
The chi- square statistic with Yates correction is 10.128. The p- value is 0.001467. Significant at p < .05 level.			

Screening of clinical samples by both PCR and LAMP

All the 250 clinical samples were screened for both Duplex PCR and LAMP as per standard protocol.

Results And Discussion

A novel nucleic acid amplification LAMP by auto cycling strand displacement DNA synthesis using *Bst* DNA polymerase large fragment was first designed by Notomi et al. 2000; Mori et al. 2001. The outer LAMP primers could anneal to the complimentary sequence of the double standard DNA in dynamic equilibrium in LAMP reaction mixture at the temperature around 65°C to initiate LAMP reaction. Parida et al. (2008) recommended that initial heat denaturation step is not required in LAMP reaction. However initial denaturation step of template DNA was added in the present study to increase the reaction efficiency as recommended by several previous workers (Notomi et al. 2000; Kamachi et al. 2006; Das et al. 2012; Radhika et al. 2016).

For standardization of LAMP different combinations of specific outer and inner primers were tried. Serial dilutions of primers with 10 p mol/μL of outer primer and the inner primers of 10 p mol/μL, 40 p mol/μL and 80 p mol/μL were tested. The outer primers concentration of 10 p mol/μL and 40 pmol/μL of inner primers (FIP and BIP) were found to be optimum for amplification of specific positive LAMP reaction in for *D. nodosus* and *F. necrophorum*. Notomi et al. (2000); Radhika et al. (2016) recommended Similar combination of outer primers at 1/4 to 1/10 concentration of the inner primers for optimal amplification. The enzyme *Bsm* polymerase large fragment with high functional similarity to *Bst* DNA polymerase was used successfully in the present study, as described by Johnson et al. (2014) to amplify *Citrus yellow mosaic badnavirus* (CMBV) by LAMP. The LAMP reaction was standardized by using different

concentrations of the enzyme 0.3 μ L, 0.5 μ L, 0.75 μ L and 1 μ L to test the optimum concentration. The enzyme concentration of 0.3 μ L was found to be optimum for positive LAMP reaction. Similarly, 0.5 μ L enzyme concentration was found to be optimum for LAMP reaction to detect *F. necrophorum* from footrot suspected sheep and goat samples. LAMP reaction was standardised at 60°C for 60 min for *D. nodosus*. In case of *F. necrophorum* the LAMP reaction was standardized at 56°C for 60 min. Similar combinations of temperature and time ranging from 60–65°C were used by several workers for successful LAMP reaction with *Bst* DNA polymerase (Notomi et al. 2000; Radhika et al. 2016; Zheney et al. 2018; Zhang et al. 2019; Wang et al. 2020).

The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR by using standard dilutions of DNA extracted from *D. nodosus* cultures. The dilutions were performed with initial ten-fold dilution followed by two-fold dilutions. LAMP and PCR was performed with all the dilutions after measuring the DNA concentration using nanodrop spectrophotometer. The PCR could detect up to the dilution of 1:320 (0.7ng/ μ L), whereas the LAMP could detect up to the dilution of 1:1280 (0.2ng/ μ L) as presented in Fig. 3. The DNA detection limit of LAMP was found to be higher than PCR. Similarly, for *F. necrophorum* LAMP the detection limit was found to be 1:1280 (0.0125ng/ μ L) of DNA dilution, whereas for PCR could detect only up to 1:320 (0.05ng/ μ L) dilution as shown in Fig. 4. Similar type of sensitivity assay of LAMP was performed by ten-fold dilutions of genomic DNA copies of target species by Sun et al. 2011 and Saxena et al. 2019.

The LAMP test results of 250 samples were compared with duplex PCR test results to determine the diagnostic sensitivity of the LAMP test. Out of 250 samples, 75 were positive for *D. nodosus* by PCR and 104 were positive for LAMP. The chi square calculated value was found to be 7.318. Similarly, for *F. necrophorum*, out of 250 samples 85 were positive by PCR and 120 were positive for LAMP as shown in Fig. 5. The chi square calculated value was 10.128 with significant level of difference between the two tests by this it could be concluded that LAMP is more sensitive than PCR in screening of the clinical samples.

Conclusion

Further validation and commercialization of the present LAMP kit for early detection of footrot got major advantage as it could be performed by non-specialist with minimal training at the field level which can avoid difficult and time consuming procedures. Screening of the samples with LAMP for both *D. nodosus* and *F. necrophorum* will help in epidemiological investigation to establish the causal association of *F. necrophorum* with *D. nodosus* in causing footrot in sheep and goat. It will also facilitate early, fast and accurate on-farm diagnosis of infection and help to reduce spread of the disease through adoption of suitable control and preventive measures including vaccination or new management strategies.

Declarations

Funding information

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Authors Contribution :

VK conceived and designed research. MK conducted experiments. VK analyzed data. MK wrote the manuscript. VK read corrected and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics Declaration : No animal ethical issues are involved in this research.

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Ethical approval : No animal ethical issues are involved in the current research.

Consent to participate : Not applicable.

Consent for publication : Not applicable.

Availability of data and material : Not applicable.

Code availability : Not applicable

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Figures

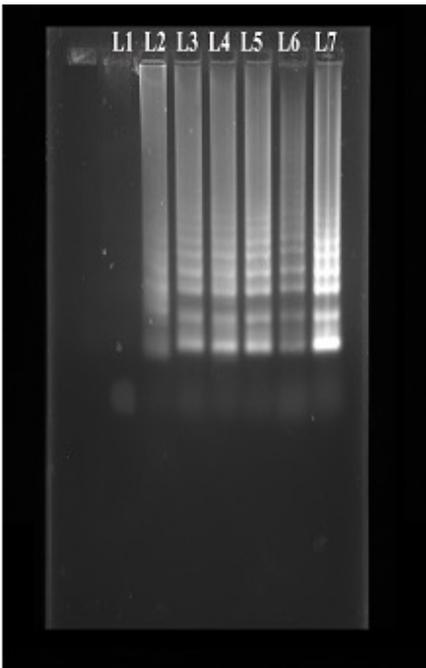


Fig 1 Screening of clinical samples for *F. necrophorum* by LAMP
L1 : Negative control L2: Positive control
L3-L7 : Field samples

Figure 1

See image above for figure legend.



**Fig. 2 Screening of clinical samples for
D. nodosus by LAMP**
L6 : Negative control
L5 : Positive control
L1-4 : Filed samples

Figure 2

See image above for figure legend.

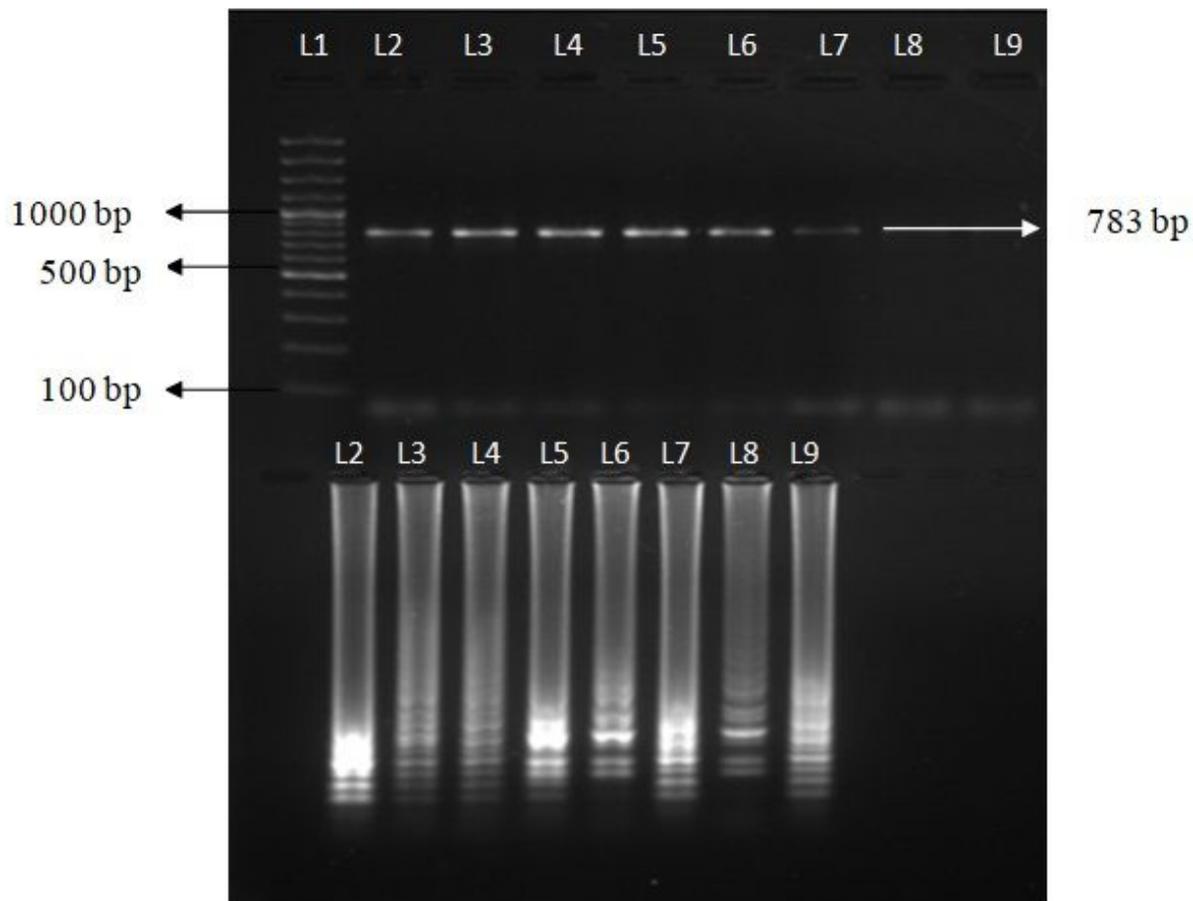


Fig 3 Comparison of efficacy LAMP with PCR targeting 16s rRNA gene of *D. nodosus* serial dilutions of Template DNA.

Upper	: PCR	Gel 2	: LAMP
L1	: 100bp ladder	L2	: positive Control
L2	: positive Control	L3-L9	: positive samples
L3-L9	: Positive samples	L10	: Negative Control
L10	: Negative Control		

Figure 3

See image above for figure legend.

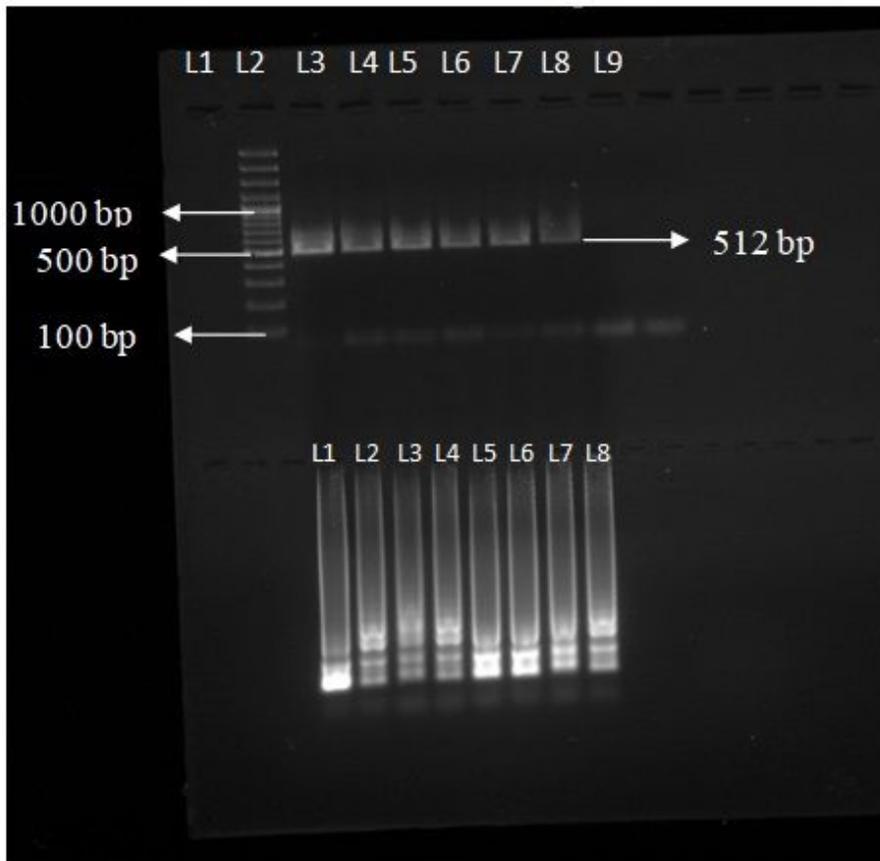


Fig 4: Comparison of efficacy of LAMP with PCR targeting *lkt A* gene of *F. necrophorum* with serial dilutions of Template DNA.

Upper	: PCR	Lower	: LAMP
L1	: Negative Control	L1	: positive control
L2	: 100bp ladder	L2-L8	: Positive samples
L3 to L8	: positive for PCR at 512 bp		
L9 and L10	: Negative for PCR		

Figure 4

See image above for figure legend.

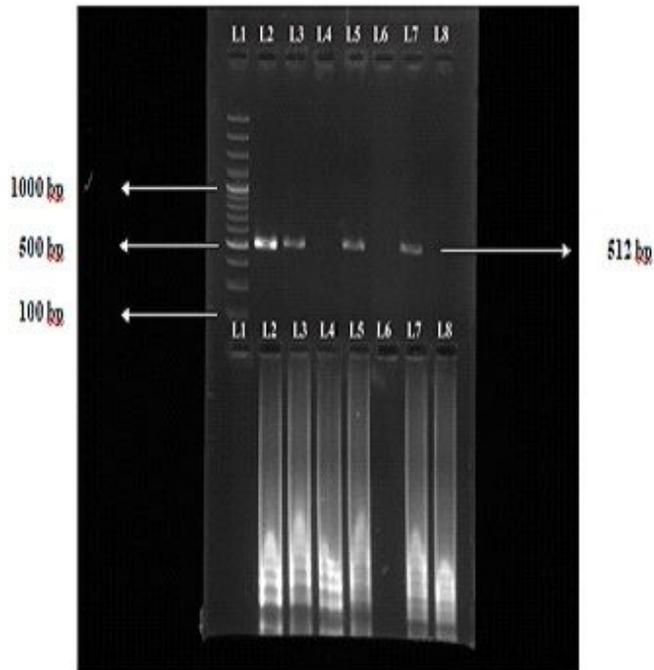


Fig. 5 Amplicons of *lktA* gene of *F. necrophorum* from clinical samples screened by both PCR and LAMP.

- | | |
|----------------------------|--|
| L1 (Upper) | : 100 bp ladder |
| L2 (Upper & Lower) | : Positive Control |
| L3, L5, L7 (Upper & lower) | : Positive for both PCR and LAMP |
| L4, L8 (Upper & lower) | : Negative for PCR and positive for LAMP |
| L6 (Upper & lower) | : Negative for both PCR and LAMP |

Figure 5

See image above for figure legend.